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Review

# The utility of ultra-high performance supercritical fluid chromatography–tandem mass spectrometry (UHPSFC-MS/MS) for clinically relevant steroid analysis

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ABSTRACT

Liquid chromatography tandem mass spectrometry (LC-MS/MS) assays are considered the reference standard for serum steroid hormone analyses, while full urinary steroid profiles are only achievable by gas chromatography (GC–MS). Both LC-MS/MS and GC–MS have well documented strengths and limitations. Recently, commercial ultra-high performance supercritical fluid chromatography–tandem mass spectrometry (UHPSFC-MS/MS) systems have been developed. These systems combine the resolution of GC with the high-throughput capabilities of UHPLC. Uptake of this new technology into research and clinical labs has been slow, possibly due to the perceived increase in complexity. Here we therefore present fundamental principles of UHPSFC-MS/MS and the likely applications for this technology in the clinical research setting, while commenting on potential hurdles based on our experience to date.

## 1. Introduction

Steroid hormones play an essential role in normal physiological function regulating a range of processes including metabolism, inflammation, immune function, salt and water balance, stress management, and reproduction. Abnormal production or metabolism of steroid hormones results in a wide array of endocrine conditions including, but not limited to, Cushing's syndrome, hyperaldosteronism, congenital adrenal hyperplasia (CAH), disorders of sexual development (DSD) and polycystic ovary syndrome (PCOS) [1]. The accurate quantification of steroid hormones is therefore vital for both research into these conditions as well as for diagnostic and monitoring purposes. These measurements are, however, complex due to the similar structure shared by all steroid hormones, precursors and metabolites. Mass spectrometry (MS) based approaches in the form of either gas chromatography–mass spectrometry (GC–MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) are currently regarded as the most accurate methods for steroid quantification [2,3]. Unlike immunoassay techniques which are hampered by cross-reactivity, MS based methods allow for accurate measurements and the simultaneous quantification of multiple steroids [4]. As a result, there is currently a drive within the endocrine community to phase out immunoassays in favour of validated MS methods. Both GC–MS and LC-MS/MS have specific strengths and limitations making the choice between them dependent on the specific application. There is now the possibility of a third option in the form of ultra-high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS). Although supercritical fluid chromatography (SFC) has been around since the 1960s, this technique has not found wide spread appeal due to numerous technical challenges associated with the use of supercritical fluids. Recent advances in technology have resulted in the development of commercial UHPSFC systems, which can potentially combine the strengths of both LC and GC, while at the same time eliminating the drawbacks of each. Arthur C. Clarke once wrote that “any sufficiently advanced technology is indistinguishable from magic”. We therefore present the basic principles of UHPSFC-MS/MS to the reader as to make this technology understandable and accessible. Furthermore, we discuss the potential use of UHPSFC-MS/MS within the clinical setting as an alternative to LC-MS/MS and GC–MS, while at the same time presenting the hurdles that remain to be overcome.

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## 2. Strengths and limitation of current MS based assays in the clinical laboratory

### 2.1. GC–MS

GC–MS achieves excellent resolution and sensitivity, and has routinely been employed for the analysis of urinary steroid metabolite profiles in patients with suspected inborn steroidogenic disorders including the variants of CAH (21-hydroxylase deficiency, 17α-hydroxylase deficiency, 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase type 2 deficiency, P450 oxidoreductase deficiency), and of other inborn steroidogenic disorders (e.g. 5α-reductase type 2 deficiency, hexose-6-phosphate dehydrogenase deficiency and cortisone reductase deficiency) [5–7]. GC–MS has also been used for prenatal screening of selected disorders using maternal urine samples [8]. In paediatrics, GC–MS is highly valued for diagnosing these rare disorders with high diagnostic sensitivity and specificity. GC–MS is also considered the mainstay discovery tool in clinical steroid investigations [5]. The advent of GC–MS/MS has further improved this cornerstone technique resulting in improvements in limits of quantitation, signal to noise and linear range [9]. Nonetheless, urine analysis by GC remains cumbersome, as it requires laborious sample preparation including enzymatic deconjugation and derivatisation, as well as solid phase and/or liquid/liquid extractions [10]. This technique is therefore expensive and time-consuming and not applicable to high-throughput assays. The time required for a result to be obtained (5–7 days) together with small batch sizes per run mean there are associated high running costs and thus preclude the use of GC–MS for screening of larger patient populations or use for routine monitoring purposes. Moreover, GC–MS cannot be used to quantify intact steroid conjugates (sulphates/glucuronides) as a deconjugation step is mandatory prior to derivatisation [3]. This obligatory deconjugation introduces the potential for incomplete deconjugation or derivatisation, as well as the potential for unwanted modifications during the hydrolysis step. Furthermore, no information pertaining to the position of the conjugate is obtainable [11–13] Bi-sulphated steroids and gluco-sulpho steroids are also not fully deconjugated and therefore not detected.

### 2.2. LC-MS/MS

Unlike GC–MS, the strength of LC-MS/MS is the specific and sensitive high-throughput analysis of several analytes [14,15]. In most cases, LC-MS/MS does not require sample derivatisation and sample preparation is typically less complex than required for GC–MS. Furthermore, ultra-high performance liquid chromatography (UHPLC) instrumentation offer significantly reduced runtimes compared to traditional LC, making high throughput assays possible. This technique is routinely used for the quantification of selected panels of serum steroids within clinical laboratories and is preferred to immuno-based assays, which are subject to cross reactivity [16]. The main drawback with short high-throughput runs is that the number of analytes that can be resolved and quantified is limited. Nonetheless, improved resolution of comprehensive steroid panels is achievable by longer chromatographic runs, which are common requirements for GC–MS methods. It should be noted that LC-MS/MS is prone to ion suppression (or enhancement) due to matrix effects. Additionally some classes of steroids, e.g. $\Delta^5$ and 5α/β-reduced steroids, ionise poorly resulting in inadequate limits of quantification [3]. Sensitivity of difficult to analyse steroids such as dehydroepiandrosterone (DHEA) and 5α-dihydrotestosterone (DHT) is improved significantly by derivatisation, but can result in increased chromatographic complexity due to the production of multiple derivative isomers [10,17]. For example, the derivatisation of the ketone group in DHEA to form DHEA-oxime improves mass spectrometry sensitivity tenfold, but produces multiple derivatives for other serum steroids such as cortisol and DHT, thus requiring further chromatographic optimisation [18,19]. Steroid conjugates can easily be quantified by LC-MS/MS, thereby making the quantification of steroids undetectable by GC–MS possible [3,20,21].

## 3. Considerations for the use of UHPSFC-MS/MS in the clinical laboratory

### 3.1. Introduction to UHPSFC-MS/MS

To cover all aspects of steroid metabolism, the steroid research laboratory should employ both LC-MS/MS and GC–MS as complimentary instruments [3]. The recent advent of commercial UHPSFC systems may, however, offer an alternative as UHPSFC combines many of the strengths of both GC and LC. SFC is itself not a new technique and was used for steroid analysis almost 30 years ago [22]. The first SFC-MS experiments were conducted by Tuomola et al., looking at androstenone in pig fat following a simple dichloromethane extraction [23]. Today SFC can be coupled to the highly sensitive, fast scanning mass spectrometer, allowing multiple low level steroids to be quantified in a single method [24]. For a comprehensive review of SFC see [2–4].

UHPSFC works through the combination of multiple solvent systems and pressure regulators. Highly miscible supercritical $CO_2$ is used as the primary mobile phase, which has liquid-like densities and dissolving capacities, but demonstrates high gas-like diffusivity and low viscosities thereby allowing for enhanced chromatographic efficiency and resolution [25,26]. Commercial UHPSFC systems make use of liquid organic modifiers of different polarities, which when combined with the supercritical $CO_2$ and wide range of stationary phases results in unparalleled versatility and selectivity [26–28]. This makes UHPSFC ideal for separating compounds with similar structures and similar mass spectra, such as steroids. While the addition of an organic modifier does technically prevent the $CO_2$ from achieving the supercritical state, the term UHPSFC is maintained as the subcritical $CO_2$ demonstrates the same advantageous mobile phase properties [29]. A back-pressure regulator is an essential component of UHPSFC systems as this prevents the decompression of the supercritical $CO_2$ during chromatographic separation (Fig. 1). While conventional UHPLC systems require a high flow rates to achieve reduced run times, the high flow rates can inhibit desolvation and suppress ionisation and therefore reduce the sensitivity of the MS [30]. During UHPSFC-MS/MS, the $CO_2$ decompresses following the post-column regulator, thereby substantially minimising the need for desolvation at the electrospray ionisation (ESI) source and enhancing ionisation. In fact, the decompression is so comprehensive, especially during conditions of low modifier content (< 5%) that the post-column addition of solvent is required to avoid analyte

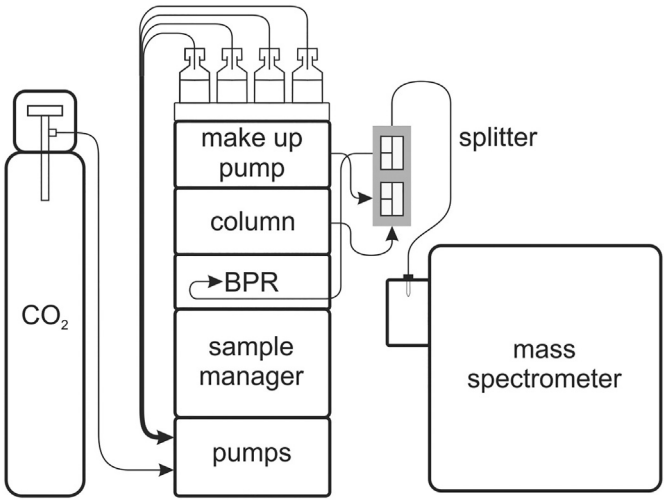


**Fig. 1.** Schematic illustrating the key elements of a UHPSFC-MS/MS system. BPR, back pressure regulator.

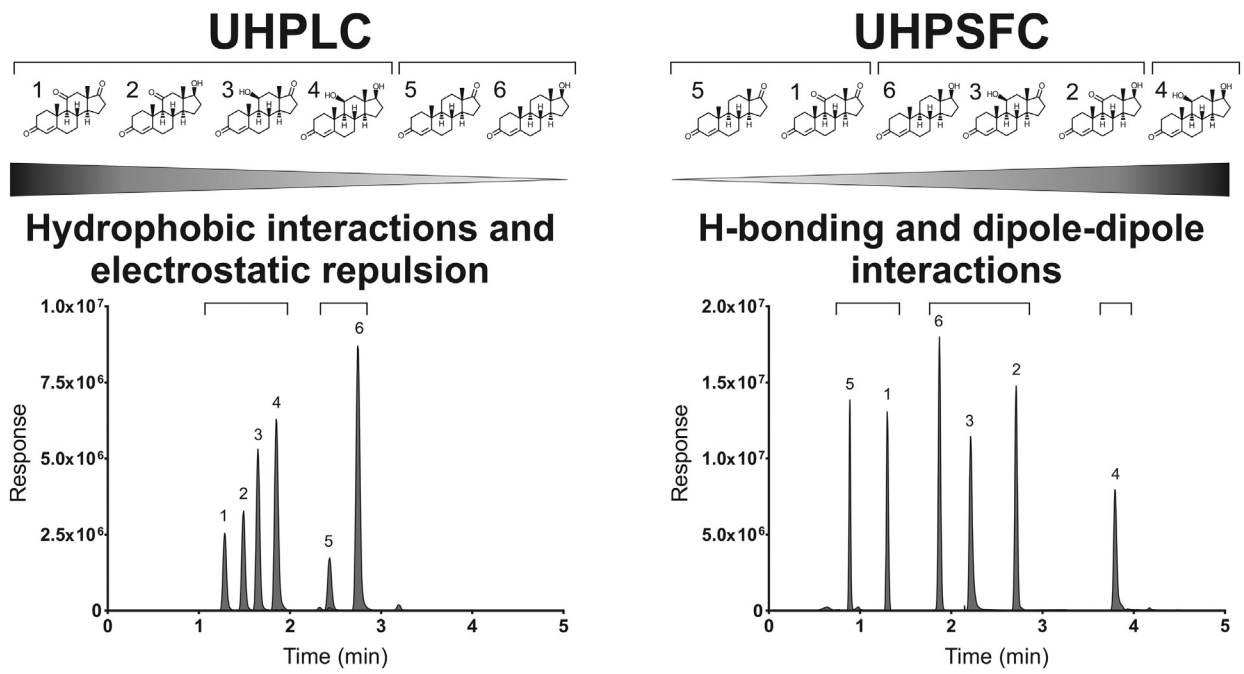


**Fig. 2.** Schematic illustrating the different separation chemistries for UHPLC and UHPSFC. During UHPLC the analytes are separated primarily based on polarity with the more polar steroids (1–4) grouping together and the less polar steroids (5 and 6) also forming a distinct grouping. During UHPSFC an orthogonal separation is observed, and three distinct groupings are observed based on hydrogen bonding potential of the steroids. Steroids: 11-ketoandrostenedione (1), 11-ketotestosterone (2), 11β-hydroxyandrostenedione (3), 11β-hydroxytestosterone (4), androstenedione (5) and testosterone (6).
Figure modified from [13].

precipitation. This post-column solvent is referred to as the make-up and is provided by an independent make-up pump (Fig. 1). In addition to preventing precipitation the make-up can also be used to aid ionisation.

An obvious advantage of this set up is that the modifier can be optimised to aid separation, while the make-up solvent and flow rate can be optimised independently to allow for optimal ionisation, thereby improving sensitivity, while having no influence on the selectivity achieved by the UHPSFC system [27,31]. Indeed, increases in sensitivity due to enhanced desolvation have been observed in studies comparing UHPLC-MS/MS to UHPSFC-MS/MS, though interestingly these effects were more pronounced in older generation triple quadrupole devices [27]. One possible explanation for this observation is that UHPSFC-MS/MS primarily makes use of organic solvents as both the modifier and make-up solvent and these are more susceptible to desolvation than water, which often forms a substantial percentage of the mobile phase used in UHPLC-MS/MS. Newer mass spectrometers may, however, be more compatible with highly aqueous mobile phases, thereby negating some of this advantage [27,32]. Although the obvious technical benefits of UHPSFC-MS/MS are clear, the implementation of any new technology is not without its challenges. We therefore discuss both the potential strength of the technique while at the same time commenting on potential hurdles.

### 3.2. Orthogonal chromatography – it's not a black box

The majority of clinicians and scientists working with steroid analyses are familiar with the elution order of steroids achieved by GC and UHPLC. With GC elution order denoted by volatility of the derivatives in the gas phase, combined with interactions within the capillary column. UHPLC and older LC methods make use of reverse phase chromatography in which separation is achieved based on polarity. Although UHPSFC is compatible with both normal and reversed phase columns, the retention mechanism is primarily based on hydrogen bonding and dipole-dipole interactions which offers an alternate, orthogonal selectivity [33]. This was recently illustrated when we compared the elution order of nineteen steroids by UHPLC and UHPSFC [24]. UHPLC with a reverse phase column clearly separated the steroids primarily based on polarity, with the more polar compounds eluting first. Conversely, separation of the same steroids by UHPSFC with a normal phase column did not yield the inverse elution sequence as might be expected for a normal phase column, but instead yielded a scrambled elution sequence that did not correlate to the results achieved by UHPLC. Although this elution order might be less intuitive to understand or predict, especially for those familiar with conventional reverse phase chromatography, a basic understanding of the retention mechanism can shed light on what might first appear to be a black box. This is illustrated well in the following example [24]. When six closely related $C_{19}$ steroids were separated by conventional reverse-phase UHPLC-MS/MS the steroids grouped well by polarity (Fig. 2). Androstenedione (A4) and testosterone (T) both contain two oxygen atoms in the form of either keto or hydroxyl groups. These steroids were the least polar and were well retained by the column. The four 11-oxygentated steroids, 11β-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4), 11β-hydroxytestosterone (11OHT) and 11-ketotestosterone (11KT) all contain three oxygen atoms resulting in the earlier elution of these four steroids which also grouped together based on the hydrophobic interactions of the steroids with the C18 carbon chain of the stationary phase. It should, however, be noted that while polarity due to this hydrophobic effect drives the distinct groupings, other factors play a role in determining the elution order within the groupings. It is clear from Fig. 2 below that within the groupings the steroids containing more keto groups elute before those with more hydroxyl groups. This may be counterintuitive as keto groups are less polar than hydroxyl groups, however, one must take the column chemistry into account. In this case separation was achieved using a High Strength Silica column (HSS) with T3 end-capping, a propriety method used by Waters. The earlier elution of steroids containing a higher proportion of less polar keto groups is likely due to electrostatic repulsion between the keto group and the oxysilane oxygen in the end cap. In this case the separation achieved by reverse-phase UHPLC can therefore be explained by a combination of hydrophobic effects (due to

differences in polarity) and electrostatic repulsion.

The scattered elution order obtained by separating the same six steroids by UHPSFC-MS/MS with a normal phase 2-ethylpyridine column can be explained by their hydrogen-bonding potential. In this instance the hydroxyl groups can act as hydrogen bond donors with the stationary phase providing the acceptor. Steroids with more hydroxyl groups are therefore retained longer on the column. A4 therefore elutes first (two keto groups), followed by 11KA4 (three keto groups). These are followed by T (one keto and one hydroxyl), 11OHA4 (two ketos, one hydroxyl), 11KT (two ketos, one hydroxyl) and 11OHT (one keto, two hydroxyls). The separation of compounds with the same number of functional groups, such as 11OHA4 and 11KT, is dependent on the location of the groups as well as additional dipole-dipole interactions. Organic modifiers and additives such as ammonium formate can act as hydrogen bond donors and/or acceptors and compete for hydrogen bonds with the stationary phase, which is accomplished by implementing a gradient from a low to high (40–50%) modifier content.

It should be noted that other column chemistries may include hydrogen bond donors or a combination of donors and acceptors and would therefore allow for altered selectivity. Available column chemistries include: Ethylene-Bridged Hybrid (BEH), BEH 2-Ethylpyridine (2-EP), Charged-Surface Hybrid Fluoro-Phenyl (PFP), High Density Diol (Diol), 2-Picolylamine (2-PIC), Diethylamine (DEA), 1-Aminoanthracene (1-AA) and Amylose-tris-(3,5-dimethylphenylcarbamate) (AMY1), among others. The advantage of this orthogonal technique is therefore the wide range of selectivity which is achieved by the compatibility of supercritical $CO_2$ with multiple organic solvents and column chemistries [26]. This makes it possible to separate steroids of similar polarities, which may not be easy to separate by UHPLC [24]. Furthermore, from our experience to date, isomers such as such as 11-ketoandrosterone and 11-ketoepiandrosterone are separated with relative ease [24]. This may be particularly useful for the high-throughput quantification of urinary steroids, which contain numerous isomers. While we have previously made use of the 2-EP column for the separation of androgens [24], preliminary screening of urinary steroids has shown that the Diol column may offer the best selectivity for this application. This is in agreement with a study which developed a screening method for doping agents, including anabolic steroids, in urine [33]. Another study has successfully demonstrated the separation of sulphated and glucuronidated steroids using 2-EP and BEH columns, respectively and included multiple sets of isomers [13].

### 3.3. Matrix effects

The coelution of substances extracted from the sample matrix with an analyte can alter the ionisation efficiency of the analyte, resulting in either signal enhancement or suppression [34]. These so called “matrix effects” therefore remain an important consideration during the development and validation of any mass spectrometry based analytical method. To date, few studies have compared matrix effects between UHPLC-MS/MS and UHPSFC-MS/MS methods and this area therefore requires substantially more work. Nonetheless, results to date suggest matrix effects are of an acceptable level or even reduced in UHPSFC-MS/MS [24,32,33,35]. When comparing matrix effects between UHPLC-MS/MS and UHPSFC-MS/MS on the quantification of 43 anabolic compounds, including anabolic steroids, from urine, Desfontaine et al. observed that UHPSFC-MS/MS resulted in substantially milder matrix effects for most compounds tested. They suggest that this phenomenon is a result of the differences in mechanisms associated with extraction and chromatography. Steroid extractions usually make use of solid phase extraction (SPE), supported liquid extraction (SLE) or liquid-liquid extraction techniques, which are all based on partition mechanism during which analytes are separated by polarity. It is therefore not surprising that during UHPLC-MS/MS, extracted matrix components often coelute with the analyte as this technique also separates the analytes based on polarity, most often using reverse-phase chromatography. Conversely, UHPSFC-MS/MS makes use of a different retention mechanism in which separation is accomplished primarily based on hydrogen bonding and dipole-dipole interactions, thus resulting in the greater likelihood of separating extracted matrix components from the analyte of interest [35]. UHPSFC-MS/MS therefore offers the potential to reduce matrix effects, however, more investigations are required for complex matrices such as serum.

### 3.4. Is sample loss due to the splitter a problem?

One of the biggest concerns expressed by clinicians or research scientists alike when learning about UHPSFC-MS/MS is the potential loss or dilution of sample due to the splitter (Fig. 1). The splitter is required to direct a substantial portion of the column effluent to the back-pressure regulator, which is an essential component of commercial UHPSFC systems. Only the remaining effluent is directed towards the MS interface. A large concern is then that valuable analytes are in fact directed away from the MS and that this causes a decrease in sensitivity. This is indeed the case when using mass-flow sensitive ionisation techniques such as atmospheric-pressure chemical ionisation (APCI). Comprehensive testing coupled to modelling revealed that the split ratio is dependent on the chromatographic conditions, including the flow rate and percentage modifier, as well as the back-pressure settings and the flow rate of the make-up solvent. Split ratios between 2 and 12 were observed depending on settings and resulted in proportional losses in sensitivity, though losses were limited to 1 order of magnitude [29]. Unlike APCI, ESI is a concentration-dependent ionisation technique. Although it may not be intuitive, the split ratio between the back-pressure regulator and MS does not influence sensitivity when using ESI as the amount of analyte lost to the back-pressure regulator is directly proportional to the amount of solvent lost and thus the concentration of the analyte directed towards the MS remains unchanged. The addition of make-up solvent does, however, dilute the analyte thereby reducing sensitivity, but comprehensive analysis has shown that this dilution factor is minimal (1.1 to 1.5) under typical conditions [29]. Therefore, although the concept of the splitter is difficult to come to terms with, especially when considering valuable clinical samples which may contain only very low levels of specific analytes, the real-world losses when using ESI are minimal. Furthermore, the increased sensitivity due to the enhanced desolvation which is achieved during UHPSFC-MS/MS seem to far outweigh any losses due to the splitter.

### 3.5. Robust sensitivity

As already mentioned a real strength of UHPSFC-MS/MS is an increase in sensitivity due to improved desolvation. We have previously demonstrated increases in sensitivity of 5-fold for the difficult to ionise steroid, DHT, and up to increases up to 50-fold for easily ionisable steroids such as T and A4 [24]. The reported increases were all despite 10-fold lower injection volume used during UHPSFC-MS/MS analysis, though it should be noted that an older generation mass spectrometer was employed for the UHPLC-MS/MS analyses. More recent comparisons of the lower limit of quantification (LLOQ) for 11-oxygenated androgens using the same mass spectrometer and MS settings revealed a 5- to 25-fold increase in sensitivity with UHPSFC-MS/MS, despite a 5-fold lower injection volume [18,24].

The same MS settings are suitable for both UHPLC and UHPSFC, with the ESI variables (desolvation temperature, desolvation gas flow and capillary voltage) having the greatest influence on UHPSFC-MS/MS sensitivity [29]. Interestingly, intentionally using suboptimal settings during UHPSFC-MS/MS reduced sensitivity 8- to 14-fold depending on the analyte tested. Losses of 45- to 215-fold were observed during UHPLC-MS/MS when the same optimal and suboptimal settings were applied, with sensitivity dropping below the LLOQ for all analytes. These results clearly show that in addition to offering increased

sensitivity, UHPSFC-MS/MS also provides robust sensitivity when compared to UHPLC-MS/MS. Notably, in both cases changes in capillary voltage had the smallest effect on sensitivity, while desolvation gas flow and desolvation temperature both had a substantial effect. This finding further demonstrates the importance of the nebulization process, which is clearly more efficient during UHPSFC-MS/MS. This is likely due to both the decompression of the $CO_2$ and the absence or reduced amount of water present in the mobile phase [29].

These benefits have also been demonstrated for the analysis of conjugated urinary metabolites [13]. Samples spiked with 1 ng/mL of conjugated steroids (glucuronidated and sulphated) were easily quantifiable by UHPSFC–MS/MS while approximately half of the tested conjugated steroids were not detectable by UHPLC–MS/MS at these levels. Moreover, the repeatability obtained using UHPSFC–MS/MS was reported to be better than that obtained by UHPLC–MS/MS.

### 3.6. Under pressure?

Although a limited number of studies to date have clearly demonstrated improved sensitivities and reduced matrix effects with UHPSFC-MS/MS as compared to UHPLC-MS/MS [29,32,33] this is not without a caveat. First it should be noted that all direct comparisons of sensitivity have been conducted using the same injection volume (typically 1 μL). From our experience injection volumes > 1–2 μL cause severe peak broadening and increases in pressure during UHPSFC-MS/MS [26], however, this can also be dependent on the width of the column. Some of the differences in sensitivity can therefore be overcome by using larger injection volumes (5–20 μL) which are better tolerated by UHPLC-MS/MS. Furthermore, the maximum operating pressure for UHPLC-MS/MS systems are usually in the region of 15,000 PSI. These systems are therefore very robust when analysing samples originating from complex biological matrixes such as serum which can lead to an increase pressure over the course of an analysis batch. The maximum operating pressure of our UHPSFC-MS/MS (Waters UPC$^2$-MS/MS) is 6000 PSI and we have experienced problems with the system exceeding the maximum pressure when analysing serum samples. Several factors therefore need to be considered when developing methods for such applications. First, the sample extraction method should be chosen to reduce the complexity of the extracted sample and minimise components which could contaminate the column and system. SLE or SPE could likely yield better results in this regard than the liquid-liquid extractions which we routinely employ when using UHPLC-MS/MS [18,36]. Second, the amount of modifier should be considered. We have found that gradients up to 40–50% modifier are required to elute some steroids, but this comes at the cost of increased pressure. Third, the flow rate should be considered – while higher flow rates reduce run times and increase throughput they also increase the pressure. Slightly lower flow rates should therefore be considered during method development to build in sufficient head room. Finally, the back-pressure regulator setting needs to be considered. Higher settings can be attractive as they increase chromatographic efficiency and reduce run times, but at the cost of increased overall operating pressure.

### 3.7. Complex method development

The potential of UHPSFC-MS/MS cannot be disputed. The development of suitable UHPSFC-MS/MS methods can, however, be more complex than that of UHPLC-MS/MS methods due to an increased number of variables which include stationary phase selection, modifier composition, flow rate, gradient, back-pressure regulator setting, make-up solvent and make-up flow rate. A good sequence to follow is presented in [33]. Briefly, a generic gradient can be used to compare different stationary phases. Methanol is a good modifier to start with. After identifying the best stationary phase, the same generic gradient can be used to test the effect of different modifiers and modifier additives. Column temperatures also need optimisation as they can affect resolution. Once the best combination of stationary, mobile phase and temperature have been identified, the gradient, flow rate and back-pressure regulator settings can be optimised individually. The make-up solvent and flow rate can subsequently be optimised.

### 3.8. Need for comprehensive cross validation with established methods/reference standards

To move from a research tool to a clinical service is not an easy route. As with all new technologies convincing clinical scientists of the benefits of changing established practice is challenging. The use of liquid chromatography mass spectrometry itself within the clinical laboratory is still relatively new, only being introduced over the last 10–20 years. Even with its many advantages mass spectrometry in a clinical laboratory is still somewhat side-lined due to the ease and usability of the large immunoassay analysers. This is, however, changing with clinicians demanding accurate quantification of multiple steroids in short time periods, which is unachievable by immunoassay [4]. The research environment has already accepted this, with mass spectrometry being recognised as the gold standard [2]. Nonetheless, the advantage must be clearly apparent before any MS based methodology is adopted. This can only be proven through cross validation to reference methods, where multiple samples are analysed and statistically correlated against currently acceptable methods. There are a few studies starting to complete this work for conventional UHPLC-MS/MS [24,29,32,35], but none yet for UHPSFC-MS/MS. Furthermore, comprehensive method validation [37] and accreditation using reference standards will be required before this technique is implemented in the clinical laboratory.

## 4. Conclusions

Given the advantages of UHPSFC-MS/MS outlined above it is clear that this technology offers an alternative to GC–MS and UHPLC-MS/MS in the clinical/research setting, possibly even bridging the gap between these two mainstay techniques. While it may be presumptuous to think that UHPSFC-MS/MS would ever replace GC–MS or UHPLC-MS/MS, this technology does offer an attractive orthogonal high throughput alternative which is well suited to routine analysis of complex steroid panels including applications such as urinary, serum and salivary steroid profiling for diagnostics and monitoring. Indeed, the simplified sample preparation and high throughput potential of this technique may greatly improve diagnostics screening methods. This may be of great value in the neonatal population as it is critically important to obtain a diagnosis of any potential endocrine disorder as early as possible as these children can present with a life threatening adrenal crisis if they are not started promptly on life-long and life-saving steroid hormone replacement treatment. The first presenting clue to a diagnosis of an inborn steroidogenesis disorder can be ambiguous genitalia from birth but patients can go on to develop adrenal crisis within the first two weeks of life. Unfortunately, with the limited availability of this modality and the time it takes from sample collection to result, GC–MS remains an inaccessible tool in the acute neonatal setting for clinicians. Consequently the mainstay for diagnosis currently remains immunoassay which carries a high false positive rate, particularly in the preterm and small for gestational age populations.

UHPSFC-MS/MS has also shown promise in the quantification of conjugated steroids, an aspect of steroid metabolomics that should continue to be explored as this has the potential to further reduce analysis times and cost [13]. As with any new technology only time and usage will reveal the true potential, but based on results thus-far UHPSFC-MS/MS has undisputedly opened new possibilities within the field of steroid analysis.

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